AP2 α is Essential for *MUC8* Expression in Human Airway Epithelial Cells

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AP2 α is Essential for *MUC8* Expression in Human Airway Epithelial Cells

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The Doctoral Dissertation Submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June 2008

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June 2008

ACKNOWLEDGEMENTS

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저에게 지금까지 그리고 이 순간에도 가장 큰 관심과 사랑으로 저를 지켜봐 주시고 믿음을 주시는 아버지, 어머님께 마음 깊이 감사 드립니다. 이제 끝이 아닌 새로 시작하는 이 시점이 언젠가 있을 멋진 인생의 결과들의 시작점이 되길 바라며, 이제 그 행로의 첫 걸음을 다시 디뎌 보려 합니다.

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Abstract

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Mucins are high molecular weight proteins that are major components of mucus. Hypersecretion of mucus is a feature of several chronic inflammatory airway diseases. *MUC8* is an important component of airway mucus, and its gene expression is up-regulated in nasal polyp epithelium. However, little is known about the mechanisms of *MUC8* gene expression. We previously demonstrated that phorbol 12-myristate 13-acetate (PMA) treatment of the airway epithelial cell line NCI-H292 increases *MUC8* gene

expression. In this study, we sought to determine which signal pathway is involved in PMA-induced *MUC8* gene expression. This study found that *MUC8* gene expression is modulated by the protein kinase C and mitogen activating protein/ERK kinase (MAPK) pathways. PD98059 (a MEK-1 inhibitor) or ERK1/2 siRNA and RO31-8200 (PKC inhibitor) significantly suppressed activator protein-2alpha (AP2α) as well as *MUC8* gene expression in PMA-treated cells. To verify the role of AP2α, we specifically knocked down the expression of AP2α with siRNA for AP2α. Significantly, knocking down of AP2α inhibited PMA-induced *MUC8* gene expression. While dominant negative forms of AP2α decreased PMA-induced *MUC8* gene expression, an over-expression of wild type AP2α increased *MUC8* gene expression in NCI-H292 cells. In addition, we identified an AP2α consensus region on the *MUC8* gene promoter. Furthermore, using lentiviral vectors for RNA interference in human nasal polyp epithelial cells, we confirmed an essential role for AP2α in *MUC8* gene expression. From

these results, we conclude that PMA induces *MUC8* gene expression through a mechanism involving PKC and MEK-ERK1/2- AP2α activation in human airway epithelial cells.

Key words: *MUC8*, Mucus hypersecretion, PMA, AP2*α*

AP2α is Essential for *MUC8* Expression in Human Airway Epithelial Cells expression

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Ⅰ. INTRODUCTION

Mucins are high molecular weight glycoproteins that are major components of the mucus. In the airway, virtually all forms of airway inflammation are associated with the overproduction of mucus, which can lead to airway obstruction. Mucins are responsible for the viscoelastic properties of secreted mucus and provide lubrication and protection for mucus membranes. Mucus hypersecretion is commonly observed in many

respiratory diseases such as rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis, and cystic fibrosis. To date, 20 distinct mucin genes have been identified. The mucins are usually subdivided into two groups based on domain: the membrane-bound and secreted mucins. MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC9, and MUC19 are secreted mucins, and MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC17, MUC18, and MUC20 are membrane-bound mucins[1-18]. The other mucin genes, including *MUC8*, have not been fully characterized.

MUC8 may be an important airway mucin, because its mRNA levels are up-regulated in middle ear effusions[19], chronic sinusitis[20], and endometrial adenocarcinomas[21]. In addition, because *MUC8* protein is over-expressed in polyp epithelium, it may be involved in chronic inflammation of the nasal mucosa. However, the mechanisms behind *MUC8* gene expression during inflammation and the signal molecules involved have not been fully elucidated.

In this study, we first observed that AP2α was over-expressed in nasal polyp epithelium. The 52-kDa transcription factor activator protein AP2α is a DNA-binding transcription factor, which regulates the expression of various genes and its activity is regulated by retinoic acid, cAMP, and phorbol esters[22, 23]. Several studies suggest that AP2α may control proliferation, apoptosis, and differentiation of mammary epithelial cells[24, 25]. In invasive carcinoma, AP2α expression in ductal epithelia is involved in the tumor process[26].

Phorbol esters such as PMA have been reported to function as tumor promoters by modulating diverse cellular responses such as gene transcription, cellular growth and differentiation, programmed cell death, immune response, and receptor desensitization through protein kinase C (PKC) signaling pathways[27, 28]. PMA can substitute for diacylglycerol, the endogenous activator of PKC, and it has been used as a model of inflammatory stimulus to study the signaling mechanisms of mucins[29-34].

PMA has been shown to upregulate several mucin genes, including MUC2, MUC5B, and MUC5AC. However, a detailed analysis of the signaling pathways involved in PMA-induced up-regulation of *MUC8* has not been performed.

Previous studies have showed that *MUC8* is clearly upregulated in nasal polyp epithelium[35-37]. In addition, inflammatory mediators such as IL-1β, tumor necrosis factor-α (TNF-α), and prostaglandin E2 promote *MUC8* expression, suggesting that the mRNA level of *MUC8* increases under inflammatory conditions. Recently, this study revealed that AP2α protein is also overexpressed in nasal polyp epithelium compared with normal nasal mucosa, indicating that this transcription factor might be important for regulating *MUC8* gene expression. To explore the biochemical mechanisms involved in AP2α and *MUC8* overexpression in nasal polyp cells, this study examined the functional significance of AP2α for *MUC8* gene transcription.

We used PMA to evaluate the signaling mechanisms between PMA-induced *MUC8* and AP2α. We found that extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) and AP2α are essential for PMA-induced *MUC8* gene expression in NCI-H292 cells. These pathways provide insight into the molecular mechanisms of mucous hypersecretion and may open up novel targets for therapeutic intervention.

Ⅱ. MATERIALS AND METHODS

1. Materials

Anti-*MUC8* antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, anti-phospho-SAPK/c-Jun NH2-terminal kinase MAP kinase (Thr183/Tyr185) antibody, and anti-human AP2α antibody were purchased from Cell Signaling (Beverly, MA, USA). RO-31-8220, PD98059 and anti-α-tubulin

antibody were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho-MEK1 (pThr386) antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Cell culture

Epithelia were isolated from scrapings of the inferior turbinate or nasal polyp. The epithelial cells were treated with 1% Pronase (Type XIV protease, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) for 18 to 20 h at 4 ℃. To remove fibroblasts, endothelial cells, and myoepithelial cells, isolated cells were placed in a plastic dish and cultured for 1 h at 37 ℃. Isolated epithelial clusters were divided into single cells by incubating them with 0.25% trypsin/EDTA. Passage-2, normal human nasal epithelial (NHNE) cells or human nasal polyp epithelial cells were seeded in 0.5 ml of culture medium onto 24.5-mm, 0.45-μm pore size Transwell clear culture inserts. Cells were cultured in a 1:1 mixture of bronchial epithelial cell growth

medium (Clonetics, CA, USA) and Dulbecco's modified Eagle's medium (Invitrogen, San Diego, CA, USA) containing all supplements. The cultures were grown submerged for the first 9 days, during which time the culture medium was changed on day 1 and every other day thereafter. The air-liquid interface (ALI) was created on day 9 by removing the apical medium and feeding the cultures only from the basal compartment. The culture medium was changed daily after creation of an ALI. Cells were treated on 7 day after confluence for all experiment, indicating that primary cells were in a differentiated state at the time of treatment. The human mucoepidermoid pulmonary carcinoma cell line NCI-H292 was purchased from the American Type Culture Collection (CRL-1848, Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) in the presence of penicillin-streptomycin (Invitrogen) at 37 ℃ in a 5% CO2 humidified chamber.

3. Treatment of cells with PMA

NCI-H292 cells were seeded into 6-well plates, cultured for 24 hr to confluency, and serum-starved in RPMI-1640 with 0.2% FBS for 24 hr. Serum-starved medium was used throughout the experiments. Cells were treated with PMA (50 ng/㎖) (Sigma-Aldrich) as indicated in each experiment. For the inhibition studies, cells were pretreated with media containing inhibitors for 30 min before it was replaced with control or PMA-containing medium. After 24 hr, cells were collected to measure *MUC8* expression.

4. Immunofluorescence microscopy

For histological study, cells were washed three times with PBS and fixed in 3% paraformaldehyde solution [3% (wt/vol) paraformaldehyde, 0.1 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 7.4, in PBS] for 10 min. The cells were then washed three times with PBS, permeabilized in 0.2% Triton®X-100/PBS for 5 min, and washed three times with PBS. Next, the cells were

blocked with 10% normal goat serum (Jackson Immuno Research Labs Inc., West Grove, PA, USA) for 1 hr, and then washed with PBS. *MUC8* proteins were detected using the *MUC8* polyclonal antibody and incubated for 24 hr at 4℃, followed by washes in PBS. This procedure was repeated with an appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100, Jackson Immuno Research labs Inc). Coverslips were mounted on the slides with Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA), and the slides were examined using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., NY, USA).

5. Immunohistochemical staining for AP2α

The procedures described in this study were approved by the Institutional Review Board of the Yonsei University College of Medicine, and all participating patients provided informed consent. Human nasal polyps were obtained from patients with chronic sinusitis, and normal nasal

mucosa specimens were obtained from healthy subjects. The nasal polyp and normal nasal mucosa specimens were fixed with 4% paraformaldehyde for 24 hr, cryoprotected with 12% and 18% sucrose, and stored in a deep freezer until use. The inserts were then divided into 10-㎛ sections, and the frozen sections were stained with anti-AP2α antibody. The reaction between the antigen and antibodies was detected using peroxidase-conjugated anti-rabbit secondary antibody. Negative controls lacked the primary antibody and used an irrelevant antibody (purified rabbit IgG were included).

6. Immunodetection and quantitation of *MUC8*

The immuno-dot blotting assay for detection of secreted and intracellular mucins produced by cultured cells has been previously described in detail[38]. Briefly, *MUC8* mucin was measured using an anti-human *MUC8* antibody. Dilutions of apical secretions, cell lysates, and standards were applied to a nitrocellulose membrane, and then incubated

with the appropriate primary antibodies, followed by a reaction with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson, PA, USA). The signal was detected by chemiluminescence (ECL kit, Amersham, Little Chalfont, UK) and a standard curve was generated by linear regression analysis from the concentration. The data are represented as mean ± standard deviation (SD) of triplicate cultures from the same experiment.

7. RNA isolation and reverse transcriptase PCR (RT-PCR)

Total RNA was extracted from cells using TRIzol® reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription reactions were performed with 3 μg of total RNA, random hexamers primers (Applied Biosystems, Foster City, CA, USA), AMV reverse transcriptase (Applied Biosystems), and RNase inhibitor (Applied Biosystems) in a final volume of 25 $\mu\ell$. The reverse transcription step ran

for 30 min at 42°C and 5 min at 95°C. RT-PCR performed with a MyCycler (Bio-Rad, CA, USA) using the primers listed in Table 1.

8. Real-time quantitative RT-PCR

TaqMan real-time quantitative RT-PCR analysis of *MUC8* gene expression was carried out with an Applied Biosystems 7300 Fast Real-Time PCR system and normalized with respect to β2-microglobulin RNA as an endogenous control. The standard curve used for quantification was generated with serial 10-fold dilutions of pGEMT-*MUC8*. The thermocycler parameters were 50 ℃ for 2 min and 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. All reactions were performed in triplicate. The relative quantity of *MUC8* mRNA was obtained using a comparative threshold method. Each reaction consisted of 12.5 $\mu\ell$ of 2X TaqMan PCR Universal PCR master mix (Applied Biosystems), 1 μg of cDNA, 800 nM of primers, and 200 nM of TaqMan hybridization probe in a

25 ㎕ volume. The probe was labeled with carboxylfluorescein at the 5'-end and quencher carboxytetramethylrhodamine at the 3'- end. Analysis of AP2α DNA quantity was performed using SYBR Green PCR Core Reagents (Applied Biosystems). For reaction, 1 ㎍ of cDNA was placed in a mixture containing 2.5 ㎕ of 10X SYBR Green buffer, 3 ㎕ of 25 mM $MgCl_2$, 2 ㎕ of 5 mM dNTP, 0.1 ㎕ of Ampli Taq Gold, 0.2 ㎕ of Amp Erase UNG (Applied Biosystems), and 800 nM of primers.

All primers and probes are listed in Table 1.

9. Western blot analyses

NCI-H292 cells were seeded into 6-well plates and cultured for 24 hr before serum starvation. After starving in 0.2 % medium for 24 hr, cells were treated with 50 ng/㎖ PMA and harvested with 2X lysis buffer (250 nM Tris-Cl, pH 6.5, 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue, and 10% glycerol). Samples (cell lysates) were resuspended in SDS-

PAGE sample buffer (50 mM Tris-HCl, pH 6.0, 10% glycerol, 2% SDS, 100 mM β-mercaptoethanol, and 0.1% bromphenol blue), boiled for 10 min, and analyzed on 12% SDS-PAGE gels. Resolved proteins were transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). Blots were blocked with 5 % skimmed milk in Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) for 1 hr at room temperature and then incubated overnight with primary antibodies (1:1000) in 0.5% Tween 20 in TBS (TTBS). After thorough washing with TTBS, the blots were further incubated for 1 hr at room temperature with horseradish peroxidase - conjugated secondary antibodies (1:2000) (Cell Signaling) in TTBS and then visualized by ECL (Amersham Biosciences).

10. Transient-transfection assay

For each transfection, NCI-H292 cells were seeded into 6-well plates and cultured for 24 hr before transfection. Cells were transfected with 1 μg of

SPRSV-AP2α (AP2α over expression), △165 (AP2α-dominant negative), or SPRSV-NN (negative control; empty vector) for 24 hr using FuGENE6 Transfection Reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions for 24 hr, treated with 50 ng/㎖ PMA for 24 hr, and then assayed by RT-PCR, real-time PCR, and western blot analysis.

Plasmids SPRSV-AP2α, △165, and SPRSV-NN were kindly provided by Dr. Trevor Williams, Yale University (New Haven, CT, USA).

11. AP2α and ERK1/2 siRNA preparation and transfection

AP2α siRNA (siAP2α) oligonucleotides (Stealth™ siRNA) were synthesized by Invitrogen. We screened AP2α mRNA (GenBank NM_001032280 and NM_003220) and selected three potential siRNA sequences with high values of knockdown probability. The siRNA sequences in NM_001032280 started at the 965th, 1238th, and 1594th bases

(965: 5'-TCGCAAGATCCTTACTCCCACGTCA-3' 1238: 5'-CCGCATG TAGA AGACCCGGGTA TTA-3' 1594: 5'-AGGGAGACGTAAAGC TGC CAACGTT-3'). Stealth RNAi Negative Control Duplex (Medium GC, Invitrogen) was used as an siRNA negative control (siCon). The siERK1 (catalog no. sc-29307), siERK2 (catalog no. sc-35335), and siRNA negative control (scrambled sequence, catalog no. sc-37007) were purchased from Santa Cruz Biotechnology. siRNA transfection into NCI-H292 cells was carried out with LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. NCI-H292 cells were seeded into 6-well plates one day before transfection and co-transfected with 15 nM of each siRNA when the cells reached 30-50% confluence.

12. Transient Transfection and Luciferase Analysis

A series of constructs of *MUC8* promoter gene were reported in our previous study[39]. NCI-H292 cells were transiently transfected with pGL3-

basic, pGL3-*MUC8* (-1644/+ 87), pGL3-*MUC8* (-1190/+ 87), pGL3-*MUC8* (-973/+ 87), and pGL3-*MUC8* (-549/+ 87) constructs using a FuGENE6 Transfection Reagent (Roche Applied Science) according to the manufacturer's instructions, incubated for 48 h, treated with 50 ng/㎖ of PMA for 24 hr, harvested, and assayed for luciferase activity using a luciferase assay system (Promega, WI, USA) according to the manufacturer's instructions. β-galactosidase activity was also assayed to standardize sample transfection efficiencies.

13. Chromatin immunoprecipitation assay (ChIP assay)

Approximately $2X10^9$ NCI-H292 cells in 150-mm dishes were treated with PBS containing 1% formaldehyde for 10 min, washed twice with PBS, and fixed with 125 mM glycine at room temperature for 5 min. The cells were rinsed twice with PBS and resuspended in 1 ml of solution A (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, and 0.5 mM EGTA)

by pipetting. After a short spin (5 min at 3000 rpm), the pellets were resuspended in solution B (10 mM HEPES, pH 6.5, 200 mM NaCl, 1 mM EDTA, and 0.5 mMEGTA) containing protease inhibitors by vigorous pipetting to extract nuclear proteins. After centrifugation at 4,000 rpm for 5 min, the nuclear pellets were resuspended in immunoprecipitation buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1% SDS, and 0.5% Empigen BB) containing protease inhibitors and sonicated to break the chromatin into fragments with an average length of 0.5-1 kb. The following antibodies were used in the assay: 2 μg of anti-AP2α antibody and 2 μg of goat anti-rabbit IgG as a negative control. The putative AP2α site (-2496 to -2297 and -2796 to -2596) and control (-5830 to -5630) primers used for ChIP analysis are listed in Table 1.

14. Electromobility shift analysis

PMA-treated NCI-H292 cells were washed with ice-cold PBS and pelleted. Pellets were resuspended in cell homogenization buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonylfluoride), incubated for 10 min on ice, and centrifuged. Cells were then resuspended in cell homogenization buffer containing 0.05% (V/V) Nonidet P-40 and then homogenized. Next, nuclei were pelleted and resuspended in cell resuspension buffer [40 mM HEPES, pH 7.9, 0.4 M KCl, 1 mM dithiothreitol, 10% (V/V) glycerol, 0.1 mM phenylmethylsulfonylfluoride, 0.1% (W/V) aprotinin, and 0.3 M NaCl]. This nuclear extract was then centrifuged at 24,000 rpm for 15 min at 4 °C, and the supernatant was aliquoted and stored at -70 °C. For the electromobility shift assay, oligonucleotides corresponding to the *MUC8* AP2α sequence (5'-GTTCTGGGGC CACTTCCCTGGCCACCG - 3') were synthesized, annealed, and end-labeled with [γ-^{32}P] ATP using T4-polynucleotidekinase (Promega). Ten micrograms of nuclear extract were incubated at room

temperature for 30 min with the ^{32}P-labeled AP2α probes in binding buffer (Promega). Oligo-nuclear protein complexes were separated from the probes by electrophoresis through 6% nondenaturing polyacrylamide gels in 0.5X Tris borate- EDTA (TBE) buffer. Supershift experiments were conducted using 2 μg of anti-phospho-AP2α antibody (Cell Signaling). The gels were dried and autoradiographed on a PhosphorImager.

15. Preparation of lentivirus shRNA

The complete details of the construction of the pLL3.7 lentivirus vector are described online (http://web.mit.edu/jacks-lab/index.html). AP2α shRNA and mutant AP2α shRNA (negative control) target sequences were 5'-GGGTATTAACATCCCAGATCA-3' and 5'- GG*A*TA*G*TA*C*CA*G*CC*T*AG*C* TC*C* -3' (italic letters represent mutated bases), respectively, which have no significant similarity to other AP2 family mRNAs. 293T cells were seeded and cultured for 24 hr until 80-90% confluency and co-transfected with

pLL3.7 and packaging vectors. The resulting supernatant was collected after 48 hr. We recovered the virus after ultracentrifugation for 5 hr at 25,000 rpm in a Beckman SW28 rotor and resuspended it in PBS (200 $\mu\ell$). Titers were determined by infecting 293T cells with serial dilutions of concentrated lentivirus. We determined the GFP expression of infected cells by flow cytometry 48 hr after infection; the titer was approximately 5 x 10^5 viral particles/$\mu\ell$.

Statistical Analysis

The statistical significance of the difference between the groups was estimated by a Wilcoxon signed-rank test.

Table 1- primers and probe used for PCR

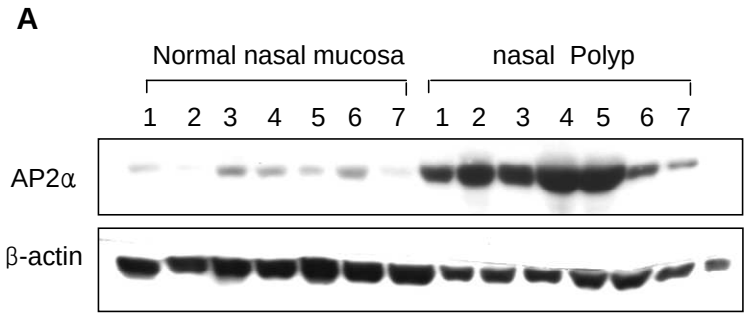
Table 1 – Primers and probe used for PCR		
Gene name	Primer sequences	Product size (bp)
Mucin 8 (RT-PCR)	Forward ACAGGGTTTCTCCTCATTG Reverse CGTTTATTCCAGCACTGTTC	239
Mucin 8 (Real Time PCR)	Forward TAACCCAATGCCACTCCTTC Reverse GGAGTGTAACCTGGCTGCTC Probe GGTTAGGGCTGACCACAGAA	202
Activator protein-2α (RT and Real time PCR) (BRIT J CANCER (2000) 82(12), 1974–1983)	Forward GCCCGTGTCCCTGTCCAA Reverse TGAGGAGCGAGAGGCGACC	132
β2-Microglobulin (RT-PCR)	Forward TCGCGCTACTCTCTCTTTCTGG Reverse GCTTACATGTCTCGATCCCACTTAA	366
β2-Microglobulin (Real Time PCR)	Forward CGCTCCGTGGCCTTAGC Reverse GAGTACGCTGGATAGCCTCCA Probe TGCTCGCGCTACTCTCTCTTTCTGGC	67
ChIP-AP2α (-2496/-2297)	Forward CGATGCGGCACCTGTTCTG Reverse ACTTTTCCAGGGGCTTGGGA	200
ChIP- AP2α (2796/-2596)	Forward TCTCAGCTGCAGTGCAGGA Reverse TCATCACACAGGCTGGCCA	201
ChIP-control (-5830/-5630)	Forward CTCAGCCTCCAGGATGCAG Reverse CCTCCCAGGGCTGTCTGT	201

III. RESULTS

1. AP2α expression in normal nasal mucosa and nasal polyp

We first examined the level of AP2α expression in normal nasal

mucosa and nasal polyp tissue by western blot analysis. AP2α was overexpressed in all nasal polyps and weakly expressed in the normal nasal mucosa (Fig. 1A). The mean AP2α expression level was 21-fold higher in nasal polyp tissue than in normal nasal mucosa (Fig. 1B). We also investigated AP2α expression in normal nasal mucosa and nasal polyps by immunohistochemical analysis (Fig. 1C). AP2α was highly expressed throughout the epithelium of nasal polyp tissue and minimally expressed in normal nasal mucosa.



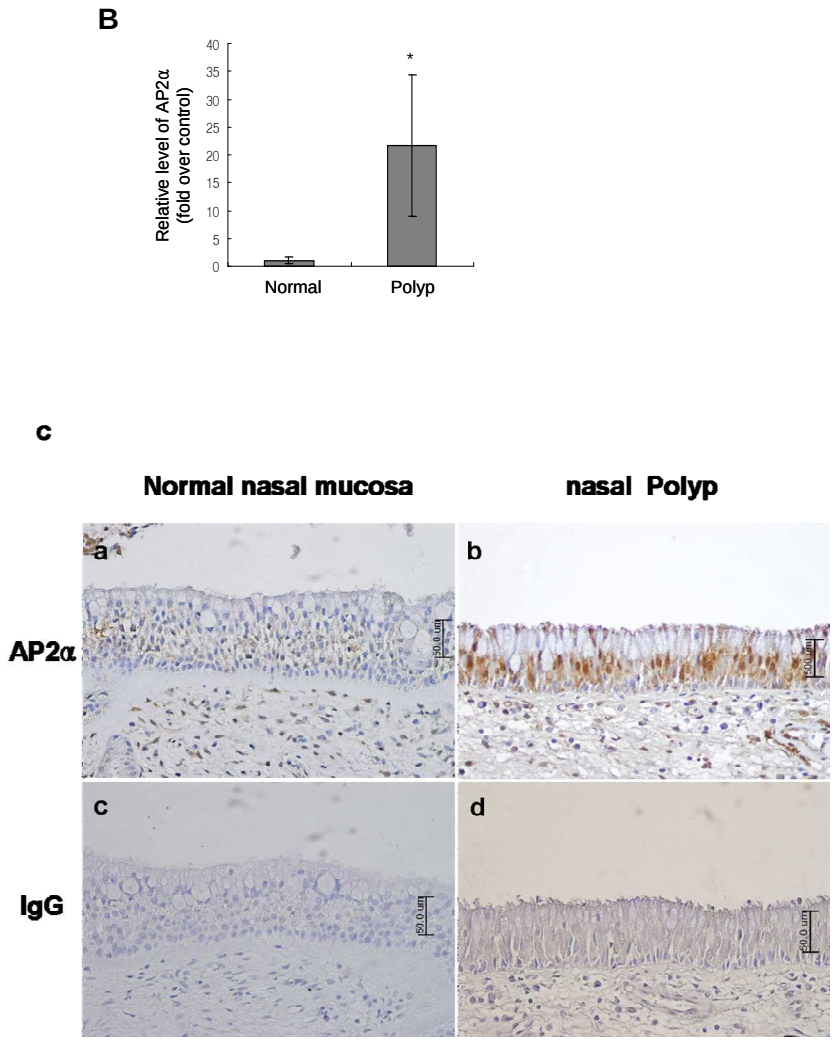


Figure 1. AP2α expression in normal nasal mucosa and nasal polyp

Normal nasal mucosa and nasal polyps were examined for AP2α expression levels. A representative western blot analysis of AP2α protein in normal nasal mucosa and nasal polyp tissue from seven patients is shown. Each lane contains the expression of β-actin used as a loading control to normalize the AP2α protein levels in each sample (A). Graph of densitometric analysis. AP2α protein band intensities were normalized by β-actin values.

Mean ± SD AP2α expression of all the nasal polyp samples normalized to β-actin and expressed as fold intensity with respect to the normal nasal mucosa (B). Immunohistochemical staining of AP2α expression in normal nasal mucosa and nasal polyps (C). Immunostaining of AP2α in normal nasal mucosa (a), and nasal polyp tissue (b). Immunostaining of IgG (negative control) in normal nasal mucosa (c), and nasal polyp tissue (d).

2. PMA up-regulates *MUC8* gene expression in NCI-H292 cells

PMA has been suggested as an inducer of *MUC2*, *MUC5AC* and *MUC5B* gene expression [6, 8-13]. In this study, we investigated the effect of PMA on *MUC8* gene expression. NCI-H292 cells were treated with 5 to 50 ng/㎖ PMA for 24 hr. PMA induced *MUC8* gene expression in a dose-dependent manner (Fig. 2A). Based on this observation, 50 ng/㎖ of PMA was used for all subsequent experiments. In addition, to determine whether PMA induced *MUC8* gene expression in a time-dependent manner, we examined *MUC8* gene expression at different time points (Fig. 2B). Stimulation with 50 ng/㎖ of PMA led to a significant time-dependent increase (with a peak at 6–24 hr after PMA stimulation). The up-regulation

of *MUC8* after PMA treatment was also observed in NCI-H292 cells using immunocytochemistry. *MUC8* expression was observed in the cytoplasm of NCI-H292 cells (Fig. 2C).

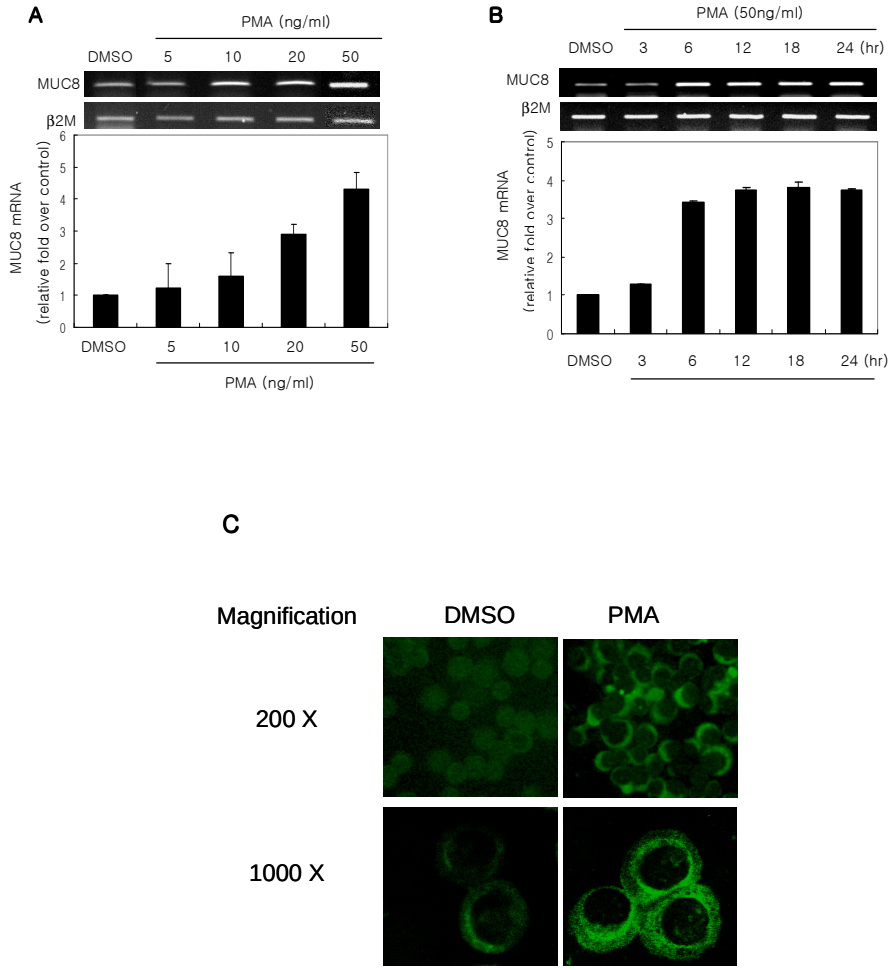


Figure 2. Effects of PMA on *MUC8* gene expression NCI-H292 cells.

Confluent cells were treated with increasing doses of PMA (5, 10, 20, and 50 ng/ml) for 24 hr (A). Confluent cells were treated with PMA (50 ng/ml) for 3, 6, 12, 18, or 24 hr (B). Total RNA was prepared and subjected to RT-PCR (upper panel) and quantitative real-time RT-PCR (lower panel) with β2-M (beta2-microglobulin) as an internal control. Twenty-four hours after treatment with PMA (50 ng/ml), cells were fixed and stained with anti-*MUC8* antibody (C).

3. AP2α is involved in PMA-induced *MUC8* gene expression.

We next evaluated AP2α mRNA expression in NCI-H292 cells stimulated with PMA for 10 min to 24 hr. AP2α mRNA expression levels peaked at 1 hr and then decreased after 12 hr (Fig. 3A). Western blot analysis of AP2α showed a marked increased in AP2α protein levels after 1 hr of treatment with PMA that decreased after 6 hr (Fig. 3B).

To determine whether AP2α plays a role in *MUC8* gene expression, siRNA was used to knockdown AP2α in NCI-H292 cells. For this purpose, NCI-H292 cells were transiently transfected with AP2α siRNA and negative control siRNA. The effects of the siRNA were analyzed by western blot analysis and RT-PCR. Quantitative real-time RT-PCR showed that cells transfected with AP2α siRNA, but not control siRNA demonstrated reduced

MUC8 gene expression (Fig. 3C). This finding indicates that AP2α is required for PMA-induced *MUC8* gene expression.

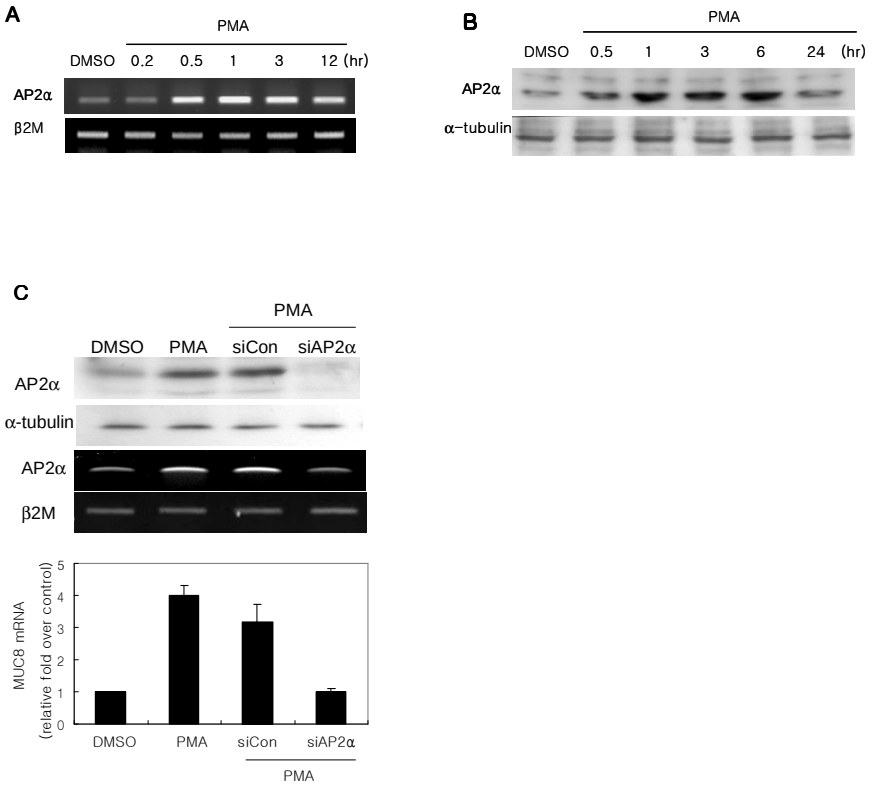


Figure 3. Involvement of AP2*α* in PMA-mediated *MUC8* gene expression in NCI-H292 cells. Confluent cells were stimulated with PMA for the indicated times, and total cell lysates were collected for AP2α RT-PCR (A) and western blot analysis (B) were performed. Cells were transiently transfected with either siAP2α or control siRNA. Twenty-four hours after transfection, the transfected cells were stimulated with PMA

for 3 hr (for Western blot analysis), 1 hr (for RT-PCR), and 24 hr (for quantitative real-time RT-PCR) (C). The figures shown are representative of three independent experiments.

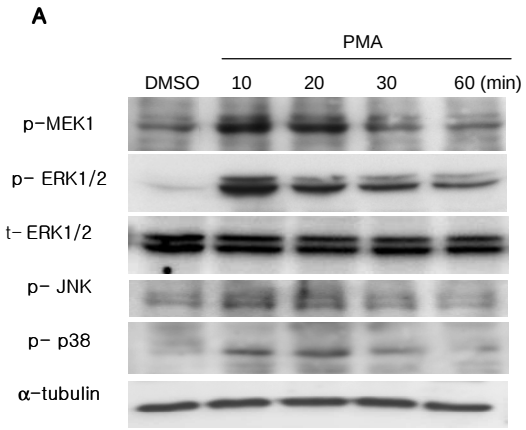
4. Involvement of MEK1-ERK1/2 MAPK in induction of *MUC8* and AP2α expression by PMA

To determine which MAPK subfamily members are actively involved in PMA-induced *MUC8* gene expression, the phosphorylation status of major MAPK members were evaluated by western blotting using phospho-specific antibodies. As shown in Fig. 4A, both MEK1 and ERK1/2 MAPK were maximally activated at 10 min, and this effect decreased after 30 and 20 min, respectively. Both c-Jun N-terminal kinase (JNK) and p38 were weakly activated at 10 min by PMA. Thus, it appears that stimulation by PMA activated the MEK1, ERK1/2, JNK, and p38 pathways in NCI-H292 cells. Because PMA significantly increased *MUC8* gene expression and MEK1, ERK1/2, and JNK activity, we investigated whether PMA-induced *MUC8* gene expression is involved in the MAP kinase pathway. To

ascertain the involvement of MEK1, ERK1/2, and JNK in PMA-induced *MUC8* gene expression, cells were exposed to PD98059 (a MEK1 inhibitor), SP600125 (a JNK inhibitor), or SB-239063 (a p38 inhibitor) before PMA treatment. Pretreatment with PD98059 for 1 hr clearly inhibited PMA-induced ERK1/2 MAPK and significantly suppressed PMA-induced *MUC8* gene expression in a dose-dependent manner (Fig. 4B). In this study, SP 600125 and SB-239063 pretreatment failed to inhibit PMA-induced *MUC8* gene expression (data not shown). To further confirm the results obtained with PD98059, NCI-H292 cells were transiently transfected with ERK1, ERK2, or negative control siRNA. In this study, PMA-induced ERK1/2 phosphorylation was lower in ERK1/2 siRNA transfected cells, which also significantly suppressed PMA-induced *MUC8* gene expression (Fig. 4C). These results demonstrate that both MEK1 and ERK1/2 MAPK are essential for PMA-induced *MUC8* gene expression in NCI-H292 cells.

Furthermore, PMA-induced AP2α expression was significantly

suppressed in cells treated with either PD98059 or transfected with ERK 1 and 2 specific siRNA. As shown in Figure 4D, PD98059 reduced the AP2α protein induced by PMA in a dose-dependent manner. Twenty-four hours after siRNA transfection, the cells were stimulated with PMA for 3 hr and then harvested for RNA. AP2α mRNA expression was suppressed by transfection with ERK1/2 siRNA but not negative control siRNA (Fig. 4E). These results indicate that both MEK1 and ERK1/2 are the main signaling molecules responsible for AP2α activation and *MUC8* gene expression in response to PMA.



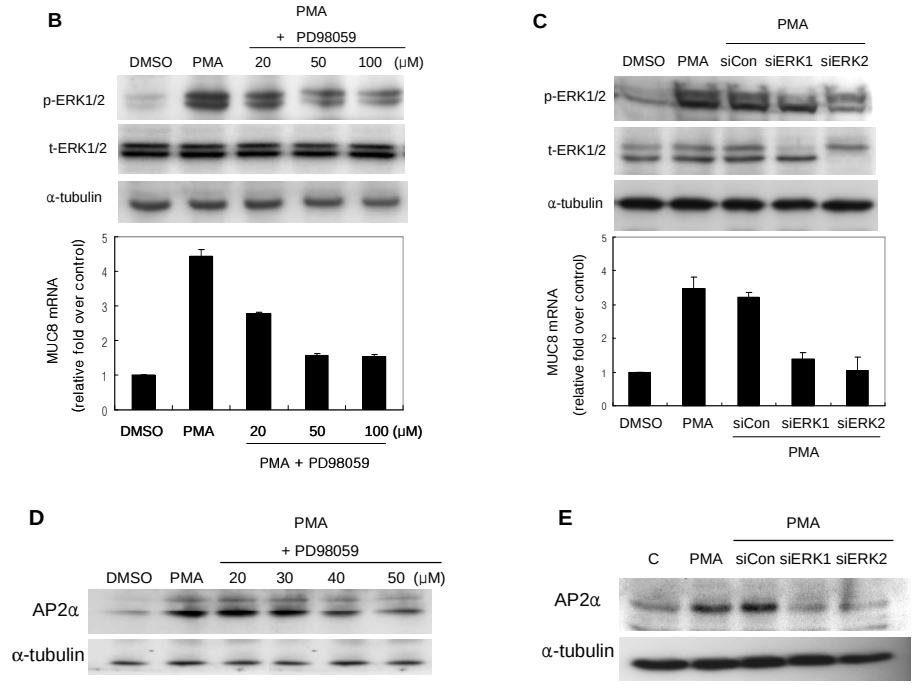


Figure 4. Involvement of MEK1-ERK1/2 MAPK in induction of MUC8 and AP2α expression by PMA. Confluent cells were treated with PMA for the indicated periods of time (10, 20, 30, or 60 min), and 30 ㎍/lane of total cell lysate protein was subjected to western blot analysis. The blotted proteins were probed with antibodies against phosphorylated or non-phosphorylated forms of MAP kinases (MEK1-ERK1/2, JNK, and p38) and α-tubulin (as a control) as indicated (A). Confluent cells were pretreated for 1 hr with PD98059 (20, 50, and 100 uM) and then stimulated with PMA for 24 hr prior to the extraction of total RNA for quantitative real-time RT-PCR. Pretreated cells were stimulated for 10 min (to detect the phosphorylation of ERK1/2; B, upper panel) or 3 hr (to detect the activation of AP2α, D) with PMA prior to the collection of cell lysate for western blot analysis. NCI-H292 cells were transiently transfected with siERK1, siERK2, or control siRNA (siCon) and stimulated with PMA for 10 min (to detect the phosphorylation of ERK1/2; C, upper panel) or 3 hr (to detect the activation of AP2α, E) prior to western blot analysis. Transfected cells were stimulated with PMA for 24 hr prior to quantitative real-time

RT-PCR (C and D, lower panel). The figures shown are representative of three independent experiments.

5. Activation of PKC mediates PMA-induced *MUC8* gene expression

To identify the molecules involved in the upstream signaling of ERK1/2 MAPK in PMA-induced *MUC8* gene expression, we investigated the role of protein kinase C (PKC) in the initiation of PMA-induced *MUC8* gene expression. PMA has been reported to induce activation of PKC in several cell types[27, 29]. PKC is known to regulate ERK1/2 activity[27, 29, and 34]. To determine the role of PKC, NCI-H292 cells were pretreated with 10 μM RO-31-8220 (a PKC inhibitor) for 1 hr before PMA stimulation, followed by western blot analysis with anti-phospho-ERK1/2 antibody (Fig 5A) or quantitative real-time RT-PCR for *MUC8* gene expression (Fig 5B). In this study, RO-31-8220 significantly inhibited PMA-induced phosphorylation of ERK1/2 and *MUC8* gene expression. These results show that PMA-induced ERK1/2 phosphorylation and *MUC8* gene expression occur via PKC activation in NCI-H292 cells.

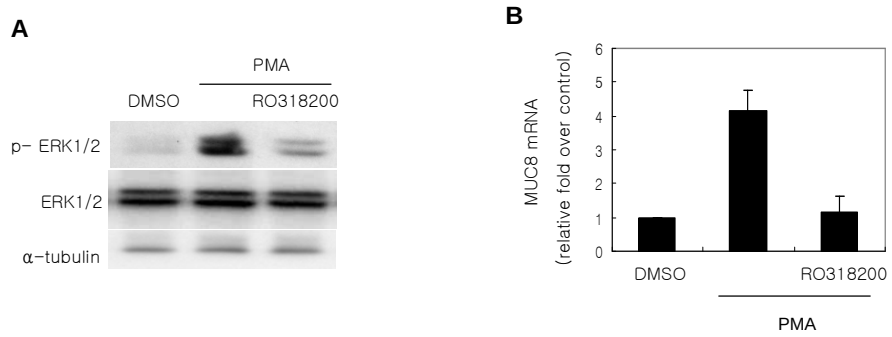


Figure 5. Activation of PKC is required for PMA-induced *MUC8* gene expression in NCI-H292 cells. Confluent cells were pretreated for 1 hr with RO-31-8220 (10 μM) and then stimulated for 10 min with PMA prior to the collection of total cell lysates for western blot analysis using anti-phospho-ERK1/2 antibody (A). After 24 hr of stimulation of inhibitor-pretreated cells, total RNA was isolated, and *MUC8* gene expression was measured using quantitative real-time RT-PCR (B). The figures shown are representative of three independent experiments.

6. Overexpression of AP2α up-regulates *MUC8* gene expression in NCI-H292 cells.

To examine the functional role of AP2α in *MUC8* gene expression, we used wild-type AP2α or mutant AP2α (dominant negative, deletion form of N-terminal amino acid) expression vectors in NCI-H292 cells. Efficient transfection of constitutively active AP2α expression (efficient expression of

AP2α) was confirmed by detection of wild-type AP2α (52 kDa) and mutant type AP2α (30 kDa) proteins (Fig. 6A). Overexpression of the wild-type AP2α gene (confirmed by RT-PCR) affected the level of *MUC8* mRNA (measured by quantitative real-time RT-PCR), suggesting that AP2α activity is required for *MUC8* gene expression. We also determined the effect of the AP2α dominant negative mutant in PMA-treated NCI-H292 cells. The mutant type AP2α vector has a DNA binding/dimerization domain, but not a transcriptional activation domain. Expression of the mutant type AP2α gene in the cells was confirmed by RT-PCR, and its inhibitory effect decreased the level of *MUC8* gene expression (measured by quantitative real-time RT-PCR, Fig. 6B). Next, we evaluated the expression level of the *MUC8* gene induced by wild type AP2α under the influence of the inhibitor PD98059. Twenty-four hours after transient transfection with wild type AP2α, NCI-H292 cells were pre-treated with the inhibitor for 1 hr and stimulated with PMA. PMA-induced AP2α and *MUC8* gene expression were inhibited by PD98059, but

not in wild-type AP2α transfected cells (Fig. 6C). These findings indicate that transfected AP2α remains in the activated state downstream of the PKC/MEK pathway and influences *MUC8* gene expression. Taken together, these results emphasize the key roles of AP2α in the transcription of *MUC8* expression in NCI-H292 cells.

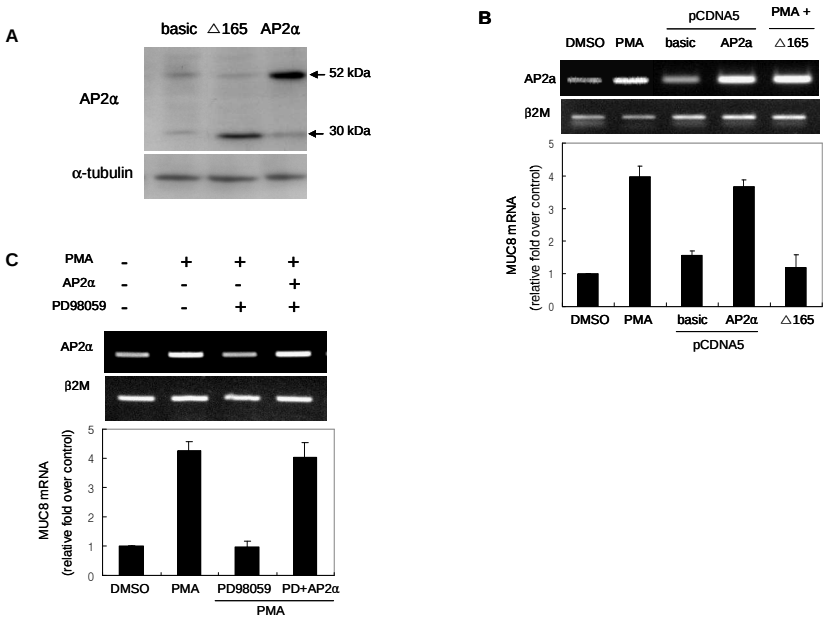


Figure 6. Overexpression of AP2α up-regulates *MUC8* gene expression in NCI-H292 cells. NCI-H292 cells were transiently transfected with basic (empty), wild-type, or mutant (△165, N terminal 165 aa deleted form) AP2α expression vectors. Western blot analysis of AP2α expression in NCI-H292 cells after gene delivery (A). Twenty-

four hours after transient transfection with the expression vector, cells were stimulated with PMA, and then total RNA was prepared for RT-PCR and quantitative real-time RT-PCR (B). Twenty-four hours after transient transfection of the expression vector, cells were pretreated with PD98059 for 1 hr, and then cells were stimulated with PMA for quantitative real-time RT-PCR (C). The figures shown are representative of three independent experiments.

7. AP2α is involved in the induction of *MUC8* promoter activity by PMA.

Systematically deleted *MUC8* 5'-flanking regions spanning from +87 bp to -1,644 bp, -1,190 bp, - 807 bp, and - 662 bp relative to the transcription initiation site were generated and cloned into the promoter less pGL3 vector. All transiently transfected *MUC8* promoter deletion constructs all showed no significant increased activity upon treatment with PMA (Fig 7A).

To further study the potential transcription factors involved in PMA-induced *MUC8* expression, the *MUC8* promoter (upstream of -1644) was analyzed for putative transcription factor binding sites with the PATCH^{TM} database (http://gene-regulation.com) and Genomatix/MatInspector database (http://genomatix.de). There are two putative AP2α binding sites (-2698/-

2684 and -2476/-2464) on the *MUC8* promoter. To assess whether AP2α directly regulates *MUC8* gene expression, we investigated which regions (transcription factor-binding sites) of the *MUC8* gene promoter were activated by PMA-induced AP2α. Since the regions -2496 to -2297 and -2796 to -2596 contain putative AP2α binding sites, we performed ChIP assays using specific primers for these regions. We also used the region -5830/-5630 as a negative control (Fig. 7B). Twenty-four hours after transfection with AP2α siRNA or control siRNA, cells were treated with PMA for 3 hr. After purification of the DNA in the immunoprecipitate, the abundance of genomic DNA containing a promoter was determined by PCR amplification using sequence-specific primer pairs. The ChIP assays clearly showed that AP2α directly binds to the *MUC8* promoter (-2496/-2297). AP2α was recruited to at the -2496/-2297 region of *MUC8* promoter upon PMA treatment. When AP2α expression was knocked down by AP2α siRNA, but not control siRNA, the recruitment of AP2α on -2496/-2297 site

was reduced. Real-time PCR was performed with -2496/-2297 region primers (Fig. 7C). Anti-rabbit IgG, the negative control, did not yield any signals, indicating the assay specificity. Input chromatins were also used in these assays to indicate that equal amounts of cell lysates were used from PMA-treated cells. Based on these results, we conclude that PMA-induced AP2α protein is strongly recruited to the chromatin regions of the AP2α binding site of the *MUC8* promoter (-2496/-2297). Consistent with this conclusion, the binding of AP2α to the *MUC8* promoter was further confirmed by electromobility shift assays using the nuclear extracts of PMA-treated NCI-H292 cells. The nuclear extract binding activities of *MUC8*-specific AP2α oligonucleotides increased remarkably in response to PMA (Fig. 7D). To identify the specific AP2α binding complex, competition and supershift analyses were performed using 50-fold excesses of non-radiolabeled AP2α oligonucleotide (cold) and anti-phospho-AP2α antibody, respectively. The intensity of specific band was selectively reduced by a

consensus AP2α competitor and anti-phospho-AP2α antibody. These results indicate that activated AP2α binds to the *MUC8* promoter.

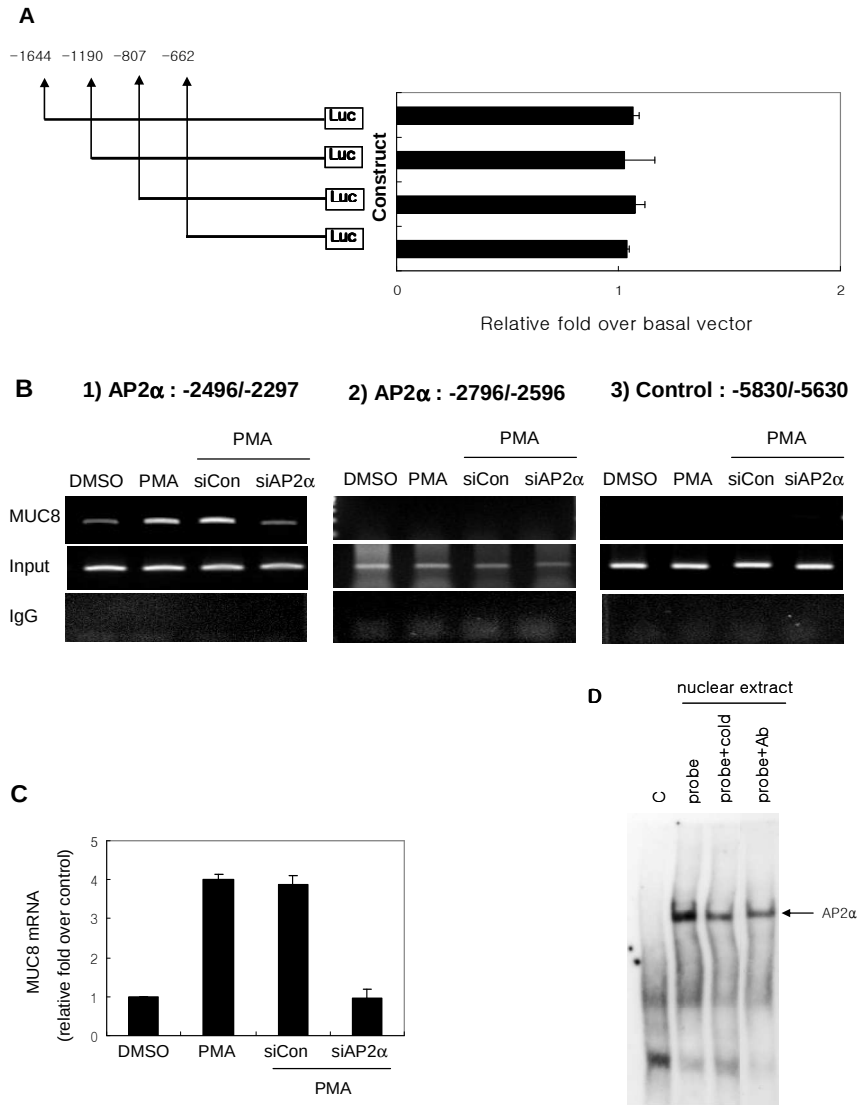


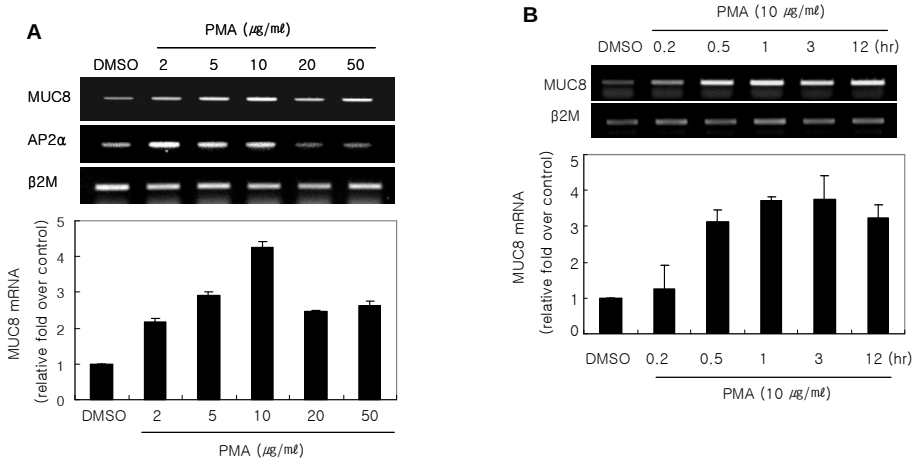
Figure 7. AP2α is involved in the induction of *MUC8* promoter activity by PMA. NCI-H292 cells were transiently transfected with deletion constructs as indicated

and then treated with PMA before harvesting for luciferase assay (A). ChIP analysis was performed with chromatin from NCI-H292 cells. Twenty-four hours after cells were transfected with siAP2α or control siRNA, they were stimulated with PMA for 3 hr. Cross-linked protein-DNA complexes were immunoprecipitated using anti-AP2α antibody or goat anti-rabbit IgG (negative control). The immunoprecipitated chromatin was analyzed with PCR using primers specific to the indicated site. Input chromatin represents a portion of the sonicated chromatin before immunoprecipitation (B). Real-time PCR was performed with -2496/-2297 region primers (C). The figures shown are representative of three independent experiments. Nuclear protein extract from PMA-treated NCI-H292 cells was subjected to an electromobility shift assay (D). Nuclear proteins were incubated with 50-fold excesses of cold probe for the competition assay and preincubated with anti-phospho- AP2α antibody for supershift before the electromobility shift assay.

8. Effects of PMA on *MUC8* gene expression in NHNE cells.

We examined whether *MUC8* is also expressed in PMA-induced normal human nasal epithelial cells (NHNE). NHNE cells were treated with 2 to 50 ng/㎖ PMA for 12 hr. The level of *MUC8* gene expression was gradually increased and reached a plateau at 10 ng/㎖, being increased by 4.3 fold (Figure 8A). Based on this observation, 10 ng/㎖ of PMA was used for all subsequent experiments in NHNE cells. In addition, to determine whether PMA induced *MUC8* gene expression in a time-dependent manner, we

examined *MUC8* gene expression at different time points (Fig. 8B). Stimulation with 10 ng/㎖ of PMA led to a significant time-dependent increase. Phosphorylation of ERK1/2, AP2 α, and *MUC8* gene expression by PMA were inhibited by pretreated with RO-31-8220 and PD98059 (Fig 8C and D), implicating PKC and MEK-ERK1/2 dependent *MUC8* gene expression in PMA-induced NHNE cells.



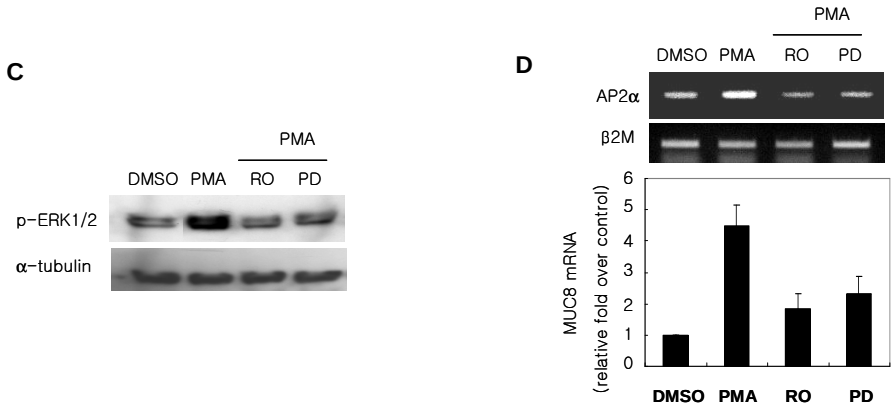


Figure 8. Effects of PMA on *MUC8* gene expression in NHNE cells. NHNE cells were treated with increasing doses of PMA (2, 5, 10, 20, and 50 ng/ml) for 12 hr (A). NHNE cells were treated with PMA (10 ng/ml) for 0.2, 0.5, 1, 3, or 12 hr (B). Total RNA was prepared and subjected to RT-PCR (upper panel) and quantitative real-time RT-PCR (lower panel) with β2-M (beta2-microglobulin) as an internal control. NHNE cells were pretreated for 1 hr with RO-31-8220 (10 μM) or PD98059 and then stimulated for 10 min with PMA prior to the collection of total cell lysates for western blot analysis using anti-phospho-ERK1/2 antibody (C). After 12 hr of stimulation of inhibitor-pretreated cells, total RNA was isolated, and *MUC8* gene expression was measured using quantitative real-time RT-PCR. The figures shown are representative of three independent experiments.

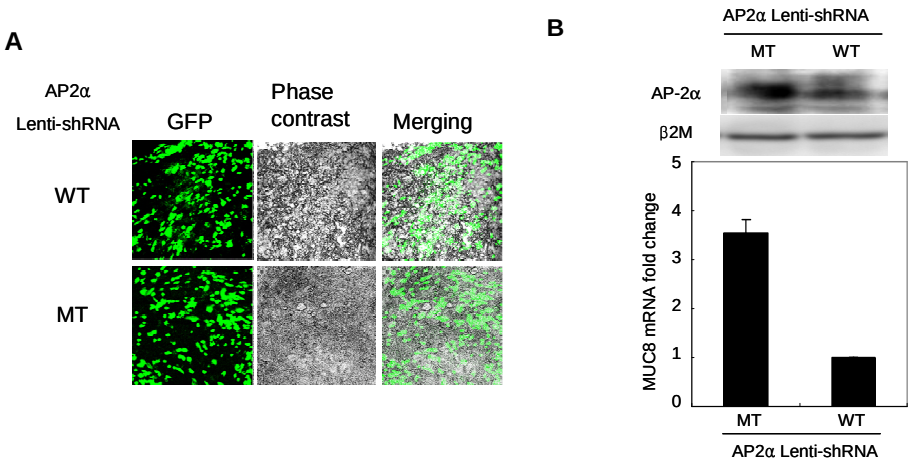
9. Silencing endogenous AP2α efficiently blocks *MUC8* gene expression in nasal polyp epithelial cells

To assess the role of AP2α in *MUC8* gene expression in nasal polyp epithelial cells, we used lentivirus shRNA to delete AP2α from the cells.

Because the AP2α siRNA sequences used in NCI-H292 cells were effective, we applied the partially overlapped siRNA sequences to the lentivirus shRNA oligo. Nasal polyp epithelial cells were infected with shAP2α lentivirus or control shRNA (mutant form of shAP2α) lentivirus before differentiation. We determined whether transgene expression could be maintained throughout the differentiation processes in nasal polyp epithelial cells. Seven days after the differentiation of cells, a GFP expression image was taken. As shown in Fig. 9A, GFP expression was easily detected in nasal polyp epithelial cells. We confirmed by western blot analysis that endogenous AP2α expression was significantly suppressed by shAP2α, whereas control shRNA did not affect endogenous AP2α expression (Fig. 9B, upper panel).

We next examined whether AP2α knockdown affects *MUC8* gene expression in nasal polyp epithelial cells. Seven days after differentiation, shRNA-infected nasal polyp epithelial cells were prepared for quantitative

real-time RT-PCR and dot-blot analysis. shAP2α markedly reduced endogenous *MUC8* gene expression (Fig. 9B, lower panel), and dot-blot analysis with anti-*MUC8* antibody on supernatants and lysates from harvested cells revealed that shAP2α decreased endogenous *MUC8* expression significantly, while control shRNA did not (Figs. 9 C and D). These results suggest that endogenously expressed AP2α is involved in *MUC8* expression.



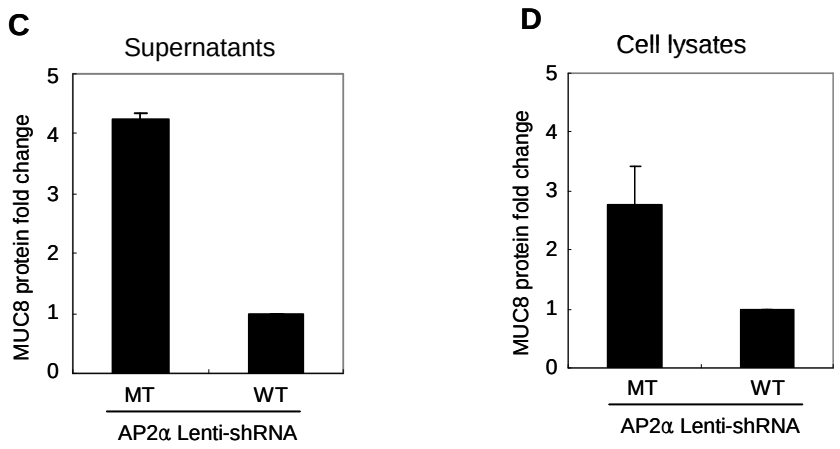


Figure 9. Lentivirus-mediated shAP2α efficiently blocks *MUC8* gene expression in nasal polyp epithelial cells. Lentivirus provides efficient delivery of exogenous genes into nasal polyp epithelial cells. Nasal polyp epithelial cells were infected with wild type AP2α lentivirus or mutant type shRNA lentivirus and then differentiated. Seven days after differentiation of cells, GFP expression and phase contrast images of the same area were taken, and the cells were lysed (7 d after differentiation) for western blot analysis of AP2α (A). Seven days after differentiation, total RNA was prepared for real-time RT-PCR (B), and supernatants (C) and cell lysates (D) were obtained for dot-blot analysis.

IV. DISCUSSION

Previous studies have suggested that *MUC8* is an important mucin gene because inflammatory mediators upregulate *MUC8* gene expression in *in vitro* culture, and *MUC8* gene expression is upregulated in human nasal

polyp epithelium. Extensive work has shown that *MUC8* may play an important role in the pathogenesis of mucus hypersecretion in polyp epithelium. Lopez-Ferrer et al. report that *MUC8* protein is overexpressed in human bronchial epithelium[40]. Moreover, Martinez-Antonet et al. indicate that the *MUC8* protein is highly expressed in both nasal polyp epithelium and submucosal glands. At the same time, the molecular mechanism of *MUC8* gene overexpression in nasal polyp epithelium remains poorly understood. In this study, we first observed AP2α expression in nasal polyp tissue and normal nasal mucosa. Since the levels of AP2α were much greater in nasal polyp epithelium, we sought to ascertain whether increased levels of AP2α might lead to *MUC8* gene expression. We found that PMA increased *MUC8* expression in NCI-H292 cells through PMA-activated AP2α. In addition, we showed that AP2α mRNA and protein were increased by PMA in a dose- and time-dependent manner, although several studies have suggested that phorbol esters, along with cyclic AMP, induce AP2α activity

independent of protein synthesis[41]. In contrast, retinoic acid induces AP2α activity by increasing AP2α mRNA levels in human teratocarcinoma cells[42, 43]. These different patterns of AP2α activity may be dependent on a cell type- and tissue-specific regulation of PMA. Further work is necessary to explore these regulatory mechanisms. Specific knockdown of AP2α with siRNA significantly reduced PMA-induced *MUC8* gene expression (Fig. 3C), showing that AP2α activation is required to induce *MUC8* gene expression in human airway epithelial cells.

PKC-mediated activation of the MEK/ERK MAPK signaling pathway and its role in regulating mucin gene expression have been studied[27, 34, 44, 45], but the exact order of events leading to AP2α activation has not been fully elucidated. To identify which MAP kinase pathways were involved in *MUC8* and AP2α expression, various kinase inhibitors were tested for their ability to reduce *MUC8* and AP2α transcription. Only activation of MEK-ERK 1/2 MAPK was required for PMA-induced *MUC8* and AP2α gene

expression, although p-38 and JNK MAPK were phosphorylated by PMA (Fig 4). Specific knockdown of ERK1 and ERK2 with siRNA completely blocked PMA-induced *MUC8* and AP2α gene expression as compared with the control, showing that the ERK1/2 MAPK activation is required to induce AP2α activation and *MUC8* gene expression in NCI-H292 cells. To confirm the involvement of AP2α in mediating the effects of PMA, we transfected cells with a dominant-negative form of AP2α and showed that PMA-induced *MUC8* gene expression was significantly inhibited by dominant-negative AP2α. In addition, overexpression of wild-type AP2α significantly increased *MUC8* gene expression (Fig. 5). These findings indicate that the expression level of *MUC8* gene is regulated by AP2α.

In the present study, our results show that the -2496 to -2297 region of the *MUC8* promoter is involved in the response to PMA and that the AP2α binding site in the -2476/-2464 region of the *MUC8* promoter is important for *MUC8* gene up-regulation by PMA.

We also tested the effect of PMA on *MUC8* gene expression in NHNE cells (Fig.8). In this study, we showed that the results in both NHNE and NCI-H292 cells were the same.

To date, the positive influence of AP2α transcription factor has been reported in mammary epithelial cells. A number of studies have reported that AP2α transcription factor has tumor suppressor activity. AP2α activity is of prognostic relevance in invasive breast cancer. The loss of AP2α expression may be associated with malignant transformation and tumor progression in malignant melanoma[46-49]. In addition, re-expression of AP2α in breast cancer and metastatic melanoma cells results in enhanced chemosensitivity, decreased tumorigenicity, and inhibited metastatic potential[35, 50]. Although much is known about the positive regulation of AP2α genes, we demonstrated in this study that AP2α transcription activity was required for *MUC8* gene expression, suggesting that AP2α may induce inflammation in airway epithelium. Furthermore, to examine whether endogenously

expressed *MUC8* in nasal polyp epithelial cells is an AP2α mediated transcription, we used lentivirus shRNA to delete AP2α from nasal polyp epithelial cells. This method is highly efficient, and because the lentivirus integrated into the cell genome[51, 52], AP2α shRNA was permanently expressed, leading to long-term knockdown of the AP2α protein. After shRNA-mediated knockdown of AP2α, *MUC8* expression was significantly lower in nasal polyp epithelial cells.

In summary, PKC-ERK1/2 MAPK signaling is essential for PMA-induced *MUC8* gene expression, and activation of AP2α is required for the intracellular mechanisms that mediate *MUC8* gene expression. AP2α was overexpressed in nasal polyp epithelial cells, and plays an important role in *MUC8* expression. From these results, we conclude that AP2α plays a crucial role in the expression of the *MUC8* gene in human airway epithelial cells.

V. CONCLUSION

In conclusion, PKC-ERK1/2 MAPK signaling is essential for PMA-induced *MUC8* gene expression, and AP2α plays a crucial role in the expression of the *MUC8* gene in human airway epithelial cells.

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국문요약

호흡기 상피 세포에서 AP2α에 의한 MUC8 발현의 조절

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문욱열

고분자 단백질체인 Mucin은 mucus의 매우 중요한 성분이며 이러한 mucus의 과 분비는 여러 가지 만성 호흡기 질환의 공통적인 특징이다. *MUC8*은 호흡기 mucus의 대표적인 mucin으로 nasal polyp 상피 세포에 과량 분포되어 있는 것이 보고되어 왔다. 그러나, 이러한 *MUC8* 유전자 발현이 유도되는 기작은 아직까지 잘 밝혀지지 않았다. 기도 상피세포 종류인 NCI-H292 세포에 Phorbol 12-myristate 13-acetate (PMA)를 처치하면 *MUC8* 유전자 발현이 증가한

다. 따라서, 우리는 염증 유도 물질인 PMA로 유도된 *MUC*8 유전자 발현에 어떠한 신호 전달 물질이 관여 하는지를 연구하였다. 이 실험에서, 우리는 *MUC8* 유전자 발현이 protein kinase C (PKC) 와 mitogen activating protein-ERK kinase (MAKP)을 통해 이루어 진다는 것을 알았다. RO31-8200 (PKC 억제물질)과 PD98059 (MEK-1 억제물질) 그리고 ERK1/2 siRNA를 이용한 실험에서, PMA로 유도된 *MUC8* 유전자뿐만 아니라 activator protein-2alpha (AP2α) 발현까지 모두 현저하게 줄어든 것을 확인 하였다. 따라서, AP2α에 대한 역할을 확인하기 위해 AP2α에 대한 siRNA를 제작 하여 실험하였고, 그 결과 PMA에 의해 증가되었던 *MUC8* 유전자가 현저하게 줄어든 것을 확인 할 수 있었다. Dominant negative 형태의 AP2α 과 발현이 PMA로 유도 되었던 *MUC8* 유전자 발현을 억제 시킨다는 것을 관찰하였고, wild 형태의 AP2α 과 발현은 *MUC8* 유전자의 증가를 유도 한다는 것을 관찰하였다. 또한, *MUC8* 유전자 발현을 조절할 수 있는 *MUC8* promoter내의 AP2α 결합 부위를 밝혀냈다. 마지

막으로 lentivirus를 이용한 AP2α 의 siRNA 실험을 nasal polyp 상피 세포를 이용하여 수행하였고, 그 결과 AP2α가 *MUC8* 유전자 발현에 매우 중요한 역할을 한다는 사실을 증명하였다. 결론적으로, 기도상피 세포내의 *MUC8* 유전자 발현은 PKC-MEK-ERK1/2-AP2α를 통한 기작으로 이루어 진다는 사실을 밝혀냈다.

핵심 되는 말: *MUC8* 유전자, 점액 과 분비, AP2α, PMA